Efficient Red-Emitting Carbon Dots and Albumin Composites for Precise Synovial Bioimaging in Rheumatoid Arthritis

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1. Materials and method

1.1 Materials

Citric acid (purity 99.5%), polyethyleneimine (Mw of 10,000 g/mol, 99%), formamide (≥99.5%), and bovine serum albumin (BSA, ≥96%) were purchased from Aladdin Reagent Company.

1.2 Preparation of CDs

Carbon dots (CDs) were synthesized via a solvothermal method. Briefly, citric acid and polyethyleneimine (Mw of 10,000 g/mol) were dissolved in formamide (30 mL) in molar ratios of 6:1 (CD1), 15:1 (CD2), 33:1 (CD3), 50:1 (CD4), respectively. The solution was then transferred to a polytetrafluoroethylene reaction vessel and reacted at 150 °C for 6 hours. Subsequently, the supernatant was collected by centrifugation and filtered through a 0.22 μm membrane filter. The resulting solution was purified by dialysis and freeze-drying to obtain a final black powder.

1.3 Synthesis of CD1@BSA

The CD1 powder was dissolved in PBS (1 mL) to prepare solutions with concentrations of 1, 2, 5 mg/mL, respectively. BSA was also dissolved in 2 mL PBS to achieve a concentration of 5 mg/mL. Subsequently, 500 μL of the CD1 solution was added to the BSA solution and vigorously stirred for 3 hours. Then, 2 mL of ethanol was added and the mixture was stirred for an additional 30 minutes. The solution was centrifuged at high speed to collect the supernatant, followed by dialysis. Finally, the CD1@BSA nanocomposites were obtained by freeze-drying.

1.4 Characterization of CD1 and CD1@BSA

The morphology of CD1 was analyzed using transmission electron microscopy (TEM). Fourier-transform infrared spectroscopy (FTIR) was employed to investigate the functional groups of CD1. Dynamic light scattering (DLS) was used to measure

the zeta potential and hydrodynamic diameter of the CD1 in aqueous solution, as well as CD1@BSA. UV-visible absorption spectra were recorded with a NanoDrop microspectrophotometer. Fluorescence emission spectra were acquired using an Edinburgh (UK) steady-state/transient fluorescence spectrometer FLS1000.

1.5 Simulation

The structure of CD1 was constructed based on XPS data. The structure of bovine serum albumin (BSA) was obtained from the Protein Data Bank (PDB ID: 3v03). Utilizing Material Studio software, we positioned five CD1 around BSA and conducted 500 iterations of structural optimization through "Geometry Optimization" to identify the conformation corresponding to the lowest energy state. The composite energy of the system was calculated using the "Energy" model. Subsequently, the energies of the pure CD1 and BSA were computed by isolating them from the composite system. Finally, the binding energy between CD1 and BSA was derived using the equation ($E = E_{A+B} - E_A - E_B$), where E represents the energy.

1.6 Cell experiments

RAW264.7 cells were cultured to 80-90% confluency and used to evaluate the cytocompatibility of CD1 and CD1@BSA via the CCK-8 assay. Solutions of CD1 at various concentrations were prepared and added to individual wells of a 96-well plate, with control groups established. The plate was then placed in a 37°C, 5% CO_2 incubator for approximately 24 hours to ensure sufficient cell exposure to the CD1. Subsequently, CCK-8 cell proliferation reagent was added to each well and further incubated for 1 hour. Absorbance analysis data for each well were then measured using a microplate reader.

To investigate the uptake of CD1 and CD1@BSA by RAW264.7 cells and the imaging functionality of CD1, RAW264.7 cells were cultured with CD1 and CD1@BSA for 12 hours. The culture medium was then aspirated, and the cells were washed three times with PBS. Under dark conditions, DAPI solution was added and incubated for 10 minutes to stain the cell nuclei. The fluorescence positions of DAPI and CD1 were analyzed to determine the intracellular distribution of CD1.

1.7 Animal experiments

All animal experiments were approved by the Animal Ethics Committee of the Model Animal Research Center of Nanjing University, with approval number IACUC-305006. Sprague-Dawley (SD) rats were used to establish collagen-induced arthritis (CIA) as an animal model of rheumatoid arthritis (RA). Initial and booster

immunizations were performed using bovine type II collagen with Freund's complete/incomplete adjuvant, administered weekly. To observe the distribution of the drugs in rat limbs, CD1 or CD1@BSA were intravenously injected, and their distribution was monitored using an in vivo imaging system (PerkinElmer, IVIS®) at 1, 6, 12, and 24 hours post-injection, with excitation and emission wavelengths of 570 and 620 nm, respectively.

1.8 Statistical analysis

All data analysis was conducted using GraphPad Prism 9.5 software. Data are presented as mean ± standard deviation. For comparisons between two groups, independent samples t-test was used after normality testing and homogeneity of variances assessment. Multiple group comparisons were performed using two-way analysis of variance (ANOVA), and Tukey test was used for pairwise multiple comparisons.

2. Result

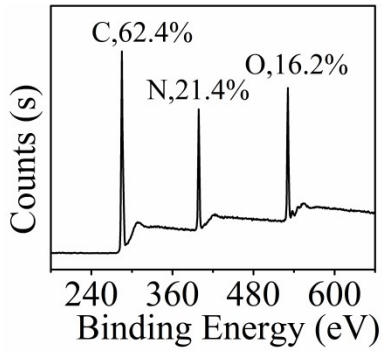

Figure S1. XPS spectrum of CD1.

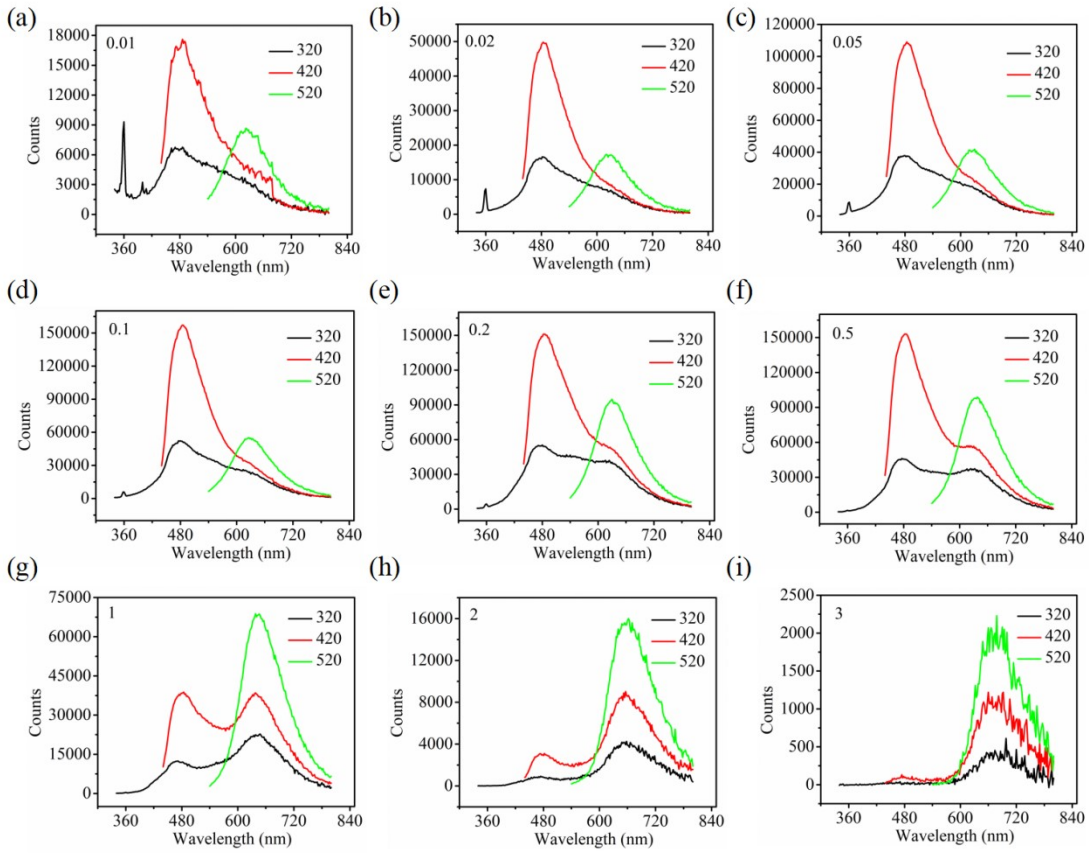

Figure S2. Emission spectra of CD1 at excitation wavelengths of 320 nm, 420 nm, and 520 nm for various concentrations of CD1: (a)-(i), 0.01-3 mg/mL.

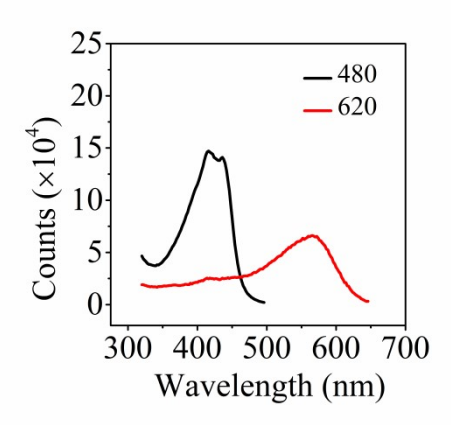

Figure S3. Excitation spectra of CD1 at 480 nm and 620 nm emission wavelengths.

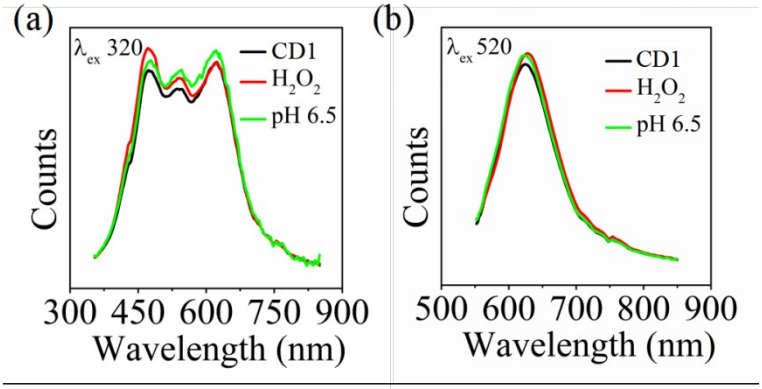

Figure S4. Fluorescence emission spectra of CD1 in H_2O_2 and pH 6.5 buffer: (a) λ_{ex}=320 nm, (b) λ_{ex}=520 nm.

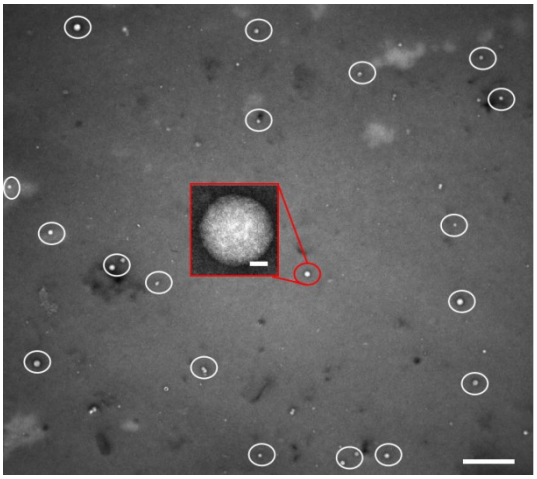

Figure S5. TEM image of CD1@BSA, scale bar = 2 μm (main image), scale bar = 20 nm (inset in the white box) .

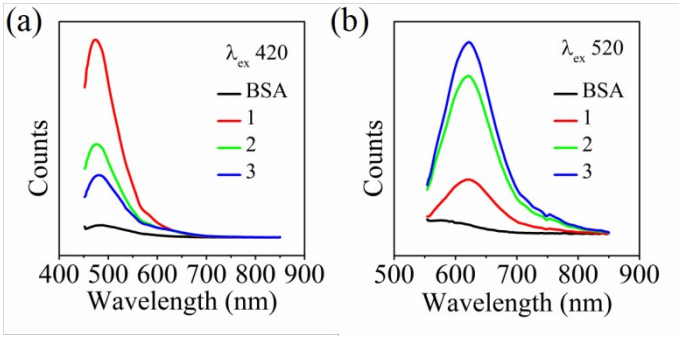

Figure S6. Emission spectra of BSA and CD1@BSA at 420 nm and 520 nm excitation wavelengths corresponding to different concentrations of CD1. 1: 1 mg/mL, 2: 2 mg/mL, 3: 5 mg/mL.

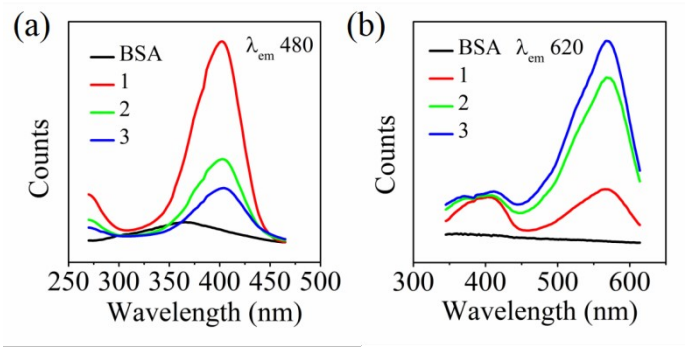

Figure S7. Excitation spectra of BSA and CD1@BSA at 480 nm and 620 nm emission wavelengths corresponding to different concentrations of CD1. 1: 1mg/mL, 2: 2mg/mL, 3: 5mg/mL.

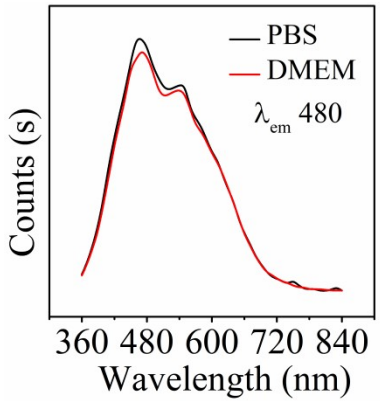

Figure S8. Excitation spectra of CD1@BSA in PBS and cell culture medium (DMEM+10% FBS).

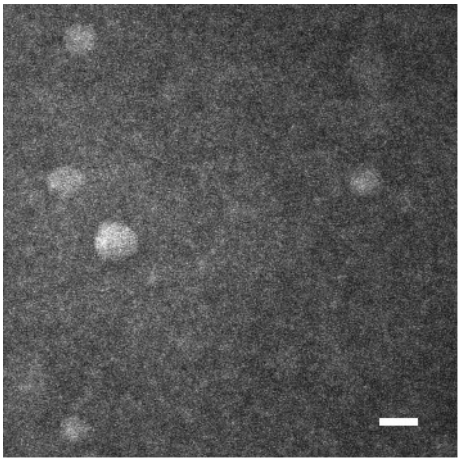

Figure S9. TEM image of CD1@BSA after treatment with cell culture medium (DMEM+10% FBS) for 24 hours, scale bar = 100 nm.